PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/62, C07K 16/24, A61K 39/395, C07K 14/715, 19/00, A61K 38/20, C07K 16/42

A1

(11) International Publication Number: WO 97/44468

(43) International Publication Date: 27 November 1997 (27.11.97)

(21) International Application Number: PCT/US97/07282

(22) International Filing Date: 16 May 1997 (16.05.97)

(30) Priority Data:
08/651,998 20 May 1996 (20.05.96) US

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(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CZ, EE, GE, HU, IL, IS, JP, KG, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TR, TT, UA, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HUMAN INTERLEUKIN-1j AND ANTAGONISTS THEREOF

(57) Abstract

Nucleic acids encoding human IL-1γ, and purified IL-1γ proteins and fragments thereof are provided. Polyclonal and monoclonal antibodies, both anti-IL-1γ antibodies and anti-idiotypic antibodies which may be agonists or antagonists of human IL-1γ, are also provided. Methods of using the compositions for both diagnostic and therapeutic utilities are also provided, together with antagonists and receptors of human IL-1γ.

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HUMAN INTERLEUKIN-1j AND ANTAGONISTS THEREOF

FIELD OF THE INVENTION

The present invention relates to compositions and methods for affecting the human immune system. In particular, it provides nucleic acids, proteins, and antibodies and other antagonists which regulate immune system response and development. Diagnostic and therapeutic uses of these materials are also disclosed.

BACKGROUND OF THE INVENTION

Recombinant DNA technology refers generally to the technique of integrating genetic information from a donor source into vectors for subsequent processing, such as through introduction into a host, whereby the transferred genetic information is copied and/or expressed in the new environment. Commonly, the genetic information exists in the form of complementary DNA (cDNA) derived from messenger RNA (mRNA) coding for a desired protein product. The carrier is frequently a plasmid having the capacity to incorporate cDNA for later replication in a host and, in some cases, actually to control expression of the cDNA and thereby direct synthesis of the encoded product in the host.

For some time, it has been known that the mammalian immune response is based on a series of complex cellular interactions, called the "immune network". Recent research has provided new insights into the inner workings of this network. While it remains clear that much of the response does, in fact, revolve around the network-like interactions of lymphocytes, macrophages, granulocytes, and other cells, immunologists now generally hold the opinion that soluble proteins, known as lymphokines, cytokines, or monokines, play a critical role in controlling these cellular interactions.

Thus, there is considerable interest in the isolation, characterization, and mechanisms of action of cell modulatory factors, an understanding of which will lead to significant advancements in the diagnosis and therapy of numerous medical abnormalities, e.g., immune system disorders.

Lymphokines apparently mediate cellular activities in a variety of ways. They have been shown to support the proliferation, growth, and differentiation of pluripotential hematopoietic stem cells into vast numbers of progenitors comprising diverse cellular lineages making up a complex immune system. Proper and balanced interactions between the cellular components are necessary for a healthy immune response. The different cellular lineages often respond in a different manner when lymphokines are administered in conjunction with other agents.

Cell lineages especially important to the immune response include two classes of lymphocytes: B-cells, which can produce and secrete immunoglobulins (proteins with the capability of recognizing and binding to foreign matter to effect its removal), and T-cells of various subsets that secrete lymphokines and induce or suppress the B-cells and various other cells (including other T-cells) making up the immune network. These lymphocytes interact with many other cell types.

Another important cell lineage is the mast cell (which has not been positively identified in all mammalian species), which is a granule-containing connective tissue cell located proximal to capillaries throughout the body. These cells are found in especially high concentrations in the lungs, skin, and gastrointestinal and genitourinary tracts. Mast cells play a central role in allergy-related disorders, particularly anaphylaxis as follows: when selected antigens crosslink one class of immunoglobulins bound to receptors on the mast cell surface, the mast cell degranulates and releases mediators, e.g., histamine,

serotonin, heparin, and prostaglandins, which cause allergic reactions, e.g., anaphylaxis.

Research to better understand and treat various immune disorders has been hampered by the general inability to maintain cells of the immune system *in vitro*. Immunologists have discovered that culturing these cells can be accomplished through the use of T-cell and other cell supernatants, which contain various growth factors, including many of the lymphokines.

Okamura, *et al.* (1995) Nature 378:88-91 describes a new cytokine that induces certain T cells to produce interferon gamma (IFN-γ), the cytokine being designated IGIF. The factor has been identified in mouse Kupffer cells and activated macrophages. No human equivalent had been described until now.

From the foregoing, it is evident that the discovery and development of new lymphokines could contribute to new therapies for a wide range of degenerative or abnormal conditions which directly or indirectly involve the immune system and/or hematopoietic cells. In particular, the discovery and development of lymphokines which enhance or potentiate the beneficial activities of known lymphokines would be highly advantageous. The present invention provides new interleukin compositions and related compounds, and methods for their use.

SUMMARY OF THE INVENTION

The present invention is directed to primate, e.g., human, interleukin-1γ (IL-1γ) and its biological activities. It includes nucleic acids coding for polypeptides themselves and methods for their production and use. The nucleic acids of the invention are characterized, in part, by their homology to cloned complementary DNA (cDNA) sequences enclosed herein, and/or by functional assays for IL-1γ activity applied to the polypeptides, which are typically encoded by these nucleic acids. Methods for modulating or intervening in the control of an immune response are provided.

The invention is based, in part, on the discovery and cloning of human cDNAs which are capable of expressing proteins having IL-1γ activity. Equivalent vectors may be constructed by using polymerase chain reaction (PCR) techniques and the sequences of the inserts.

The invention provides *inter alia* a substantially pure primate IL-1γ, a fusion protein comprising primate IL-1γ sequence, an antibody specific for binding to a primate IL-1γ, and a nucleic acid encoding a human IL-1γ or fusion protein thereof.

In the substantially pure IL-1γ embodiment, the IL-1γ may comprise a mature sequence contained within the amino acid sequence defined by SEQ ID NO: 2. The latter sequence contains a signal sequence which is not a typical signal sequence but which, like a prosequence analogous to the IL-1b prodomain that is cleaved by a convertase-like enzyme, is likely to run from the amino acid position 1 (met) to about 36 (asp). See Dinarello (1994) FASEB J. 1314-1325. The mature protein should begin at about 37 (tyr). Alternativley, the IL-1γ may exhibit a post-translational modification pattern distinct from that of natural IL-1γ. In another embodiment, the composition will comprise the primate IL-1γ and a pharmaceutically acceptable carrier. Generally, the IL-1γ can induce production of IFN-γ by a T cell or NK cell, alone or in combination with IL-12 or IL-2.

Fusion proteins of the invention comprise IL-1γ or a substantial fragment thereof covalently linked to a binding partner. In one fusion protein embodiment, the protein may comprise a sequence of SEQ ID NO: 2 and/or sequence of another cytokine or chemokine. Such protein may comprise a modification in sequence corresponding to an amino acid residue of SEQ ID NO: 2 at positions 88 (tyr) to 96 (met); or may exhibit IL-1γ agonist activity, and comprise a substitution in sequence corresponding to an amino acid residue of SEQ ID NO: 2 at positions 37 (tyr) to 82 (ile) or 102 (val) to 191 (asn).

In another fusion protein embodiment the pharmacokinetic half-life of the IL-1γ or fragment thereof is increased by conjugation to another polypeptide, e.g., to part of an immunoglobulin (Ig) chain, preferably a constant region (Fc), or to a polyethylene glycol (PEG) molecule(s), sometimes referred to as pegylation. Such fusion proteins may be referred to as IL-1γ-Ig and PEG-IL-1γ, respectively. Methods for linking polyethylene glycol (PEG) groups and Ig chains, parts thereof or other polypeptides to proteins are well known in the art.

See, e.g., International Application Publication No. WO 96/18412, which describes the fusion of polypeptides derived from immunoglobulin chains to a variety of cytokines to increase the circulating half-life of the cytokines. Methods for conjugating PEG to proteins have been described, e.g., by Davis *et al.* (U.S. Patent No. 4,179,337), Nakagawa *et al.* (U.S. Patent No. 4,791,192), and Nitecki *et al.* (U.S. Patent No. 4,902,502).

In certain antibody embodiments, the IL-1γ is a human protein; the antibody is raised against a peptide sequence of SEQ ID NO: 2; the antibody is raised to a purified primate IL-1γ; the antibody is a monoclonal antibody; or the antibody is labeled.

In various nucleic acid embodiemnts, the IL-1γ is from a human; the nucleic acid (e.g., SEQ ID NO: 1) encodes a peptide sequence of SEQ ID NO: 2; the nucleic acid is an expression vector; or the nucleic acid comprises a deoxyribonucleic acid nucleotide.

The present invention also embraces a kit comprising: a substantially pure primate IL-1γ, or fragment thereof; an antibody which specifically binds a primate IL-1γ; or a nucleic acid encoding a human IL-1γ or peptide. Various of these kits will be capable of making a qualitative or quantitative analysis.

Another embodiment of the invention includes a method of modulating physiology or development of a cell comprising contacting the cell, or an immune system

containing the cell, with an agonist or antagonist of a primate IL-1γ. This includes methods where the contacting is in combination with IL-2 and/or IL-12. Alternatively, the invention provides a method where the contacting is with an antagonist, e.g., an antibody against a primate IL-1γ, and may be combined with antagonists to IL-2 and/or IL-12. Often, the modulating is regulation of IFN-γ production; and includes contacting is with an agonist of a human IL-1γ. In other embodiments, the modulating is regulation of: an infectious disease; a vaccine response; an allergic reaction; a T helper mediated response; or a cancer.

DESCRIPTION OF THE INVENTION

All references cited herein are hereby incorporated in their entirety by reference.

The present invention provides the amino acid sequence and DNA sequence of human interleukin molecules having particular defined properties, both structural and biological, designated herein as human interleukin-1γ (IL-1γ). A cDNA encoding this molecule was obtained from an activated human monocyte cDNA library, designated M1.

Some of the standard methods are described or referenced, e.g., in Maniatis, *et al.* (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor Press; Sambrook, *et al.* (1989) Molecular Cloning: A Laboratory Manual, (2d ed.), vols 1-3, CSH Press, NY; Ausubel, *et al.*, Biology, Greene Publishing Associates, Brooklyn, NY; or Ausubel, *et al.* (1987 and periodic supplements) Current Protocols in Molecular Biology, Greene/Wiley, New York; all of which are each incorporated herein by reference.

Isolation of the human gene solved uncertainties which make its isolation far from certain. These uncertainties include: (1) whether a human counterpart of the mouse protein exists; (2) the level of similarity of the encoding nucleic acid sequences; (3) where in the sequence homology exists, useful in a PCR approach;

(4) the level of expression, thereby providing a useful source for isolating a natural cDNA gene portion; (5) cross-species biological activity, useful, e.g., in a context of expression cloning using mouse cells; and (6) immunological antibody cross reaction, important in an antibody binding based approach. Many other problems exist in successfully deriving a cross-species isolation.

A complete nucleotide sequence (SEQ ID NO: 1) and corresponding amino acid sequence (SEQ ID NO: 2) of the human IL-1γ coding segment is provided in the Sequence Listing. The human isolate shows homology to the mouse counterpart, about 71% identity at the nucleotide level; and about 65% at the amino acid level.

As used herein, the term IL-1γ shall be used to describe a protein comprising a protein or peptide segment having the mature amino acid sequence shown in SEQ ID NO: 2, or a substantial fragment thereof. The invention also includes a mutein agonist or antagonist. Typically, such agonists exhibit less than about 10% sequence differences, and thus will often have between 1- and 11-fold substitutions. It also encompasses allelic and other variants, e.g., natural polymorphic, of the protein described. Typically, it will bind to its corresponding biological receptor with high affinity, e.g., at least about 100 nM, usually better than about 30 nM, preferably better than about 10 nM, and more preferably at better than about 3 nM. The term shall also be used herein to refer to related naturally occurring forms, e.g., alleles, polymorphic variants, and metabolic variants of the human protein.

This invention also encompasses proteins or peptides having substantial amino acid sequence homology with the amino acid sequence in SEQ ID NO: 2, but excluding any protein or peptide which exhibits substantially the same or lesser amino acid sequence homology than does the corresponding IGIF protein found in the mouse. It will include sequence variants with relatively few substitutions, e.g., preferably less than about 3-5.

A substantial polypeptide "fragment", or "segment", is a stretch of amino acid residues of at least about 8 amino acids, generally at least 10 amino acids, more generally at least 12 amino acids, often at least 14 amino acids, more often at least 16 amino acids, typically at least 18 amino acids, more typically at least 20 amino acids, usually at least 22 amino acids, more usually at least 24 amino acids, preferably at least 26 amino acids, more preferably at least 28 amino acids, and, in particularly preferred embodiments, at least about 30 or more amino acids. Sequences of segments of different proteins can be compared to one another over appropriate length stretches.

Amino acid sequence homology, or sequence identity, is determined by optimizing residue matches, if necessary, by introducing gaps as required. See, e.g., Needleham, *et al.*, (1970) J. Mol. Biol. 48:443-453; Sankoff, *et al.*, (1983) chapter one in Time Warps, String Edits, and Macromolecules: The Theory and Practice of Sequence Comparsion, Addison-Wesley, Reading, MA; and software packages from IntelliGenetics, Mountain View, CA; and the University of Wisconsin Genetics Computer Group (GCG), Madison, WI; each of which is incorporated herein by reference. This changes when considering conservative substitutions as matches. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

Homologous amino acid sequences are intended to include natural allelic and interspecies variations in the cytokine sequence. Typical homologous proteins or peptides will have from 50-100% homology (if gaps can be introduced), to 60-100% homology (if conservative substitutions are included) with an amino acid sequence segment of SEQ ID NO: 2. Homology measures will be at least about 70%, generally at least 76%, more generally at least 81%, often at least 85%, more often at least

88%, typically at least 90%, more typically at least 92%, usually at least 94%, more usually at least 95%, preferably at least 96%, and more preferably at least 97%, and in particularly preferred embodiments, at least 98% or more. The degree of homology will vary with the length of the compared segments. Homologous proteins or peptides, such as the allelic variants, will share most biological activities with the embodiment described in SEQ ID NO: 2. Preferably a related polypeptide will contain a plurality of such matching fragments, e.g., at least 2, preferably 3, 4, 5, or more of specific, or assorted, lengths.

As used herein, the term "biological activity" is used to describe, without limitation, synergistic induction by splenocytes of IFN-γ in combination with IL-12 or IL-2, with or without anti-type I or anti-type II IL-1 receptor antibodies, or more structural properties as receptor binding and cross-reactivity with antibodies raised against the same or a polymorphic variant of the described human IL-1γ.

The terms ligand, agonist, antagonist, and analog include molecules that modulate the characteristic cellular responses to IL-1γ or IL-1γ-like proteins, as well as molecules possessing the more standard structural binding competition features of ligand-receptor interactions, e.g., where the receptor is a natural receptor or an antibody. The cellular responses likely are mediated through binding of IL-1γ to cellular receptors distinct from the type I or type II IL-1 receptors. Also, a ligand is a molecule which serves either as a natural ligand to which said receptor, or an analog thereof, binds, or a molecule which is a functional analog of the natural ligand. The functional analog may be a ligand with structural modifications, or may be a wholly unrelated molecule which has a molecular shape which interacts with the appropriate ligand binding determinants. The ligands may serve as agonists or antagonists, see, e.g., Goodman, *et al.* (eds) (1990)

Goodman & Gilman's: The Pharmacological Bases of Therapeutics, Pergamon Press, New York.

Rational drug design may also be based upon structural studies of the molecular shapes of a receptor or antibody and other effectors or ligands. Effectors may be other proteins which mediate other functions in response to ligand binding, or other proteins which normally interact with the receptor. One means for determining which sites interact with specific other proteins is a physical structure determination, e.g., x-ray crystallography or 2 dimensional NMR techniques. These will provide guidance as to which amino acid residues form the molecular contact regions. For a detailed description of protein structural determination, see, e.g., Blundell and Johnson (1976) Protein Crystallography, Academic Press, New York.

The human IL-1γ protein has a number of different biological activities. The human IL-1γ is homologous to the mouse IGIF protein, but has structural differences. For example, the human IL-1γ gene coding sequence has only about 71% homology with the nucleotide coding sequence of mouse IGIF. At the amino acid level, there is about 64% identity. This level of similarity suggests that the new IL-1γ protein is related to the other IL-1α and IL-1β.

The mouse IGIF molecule has rather minimally defined biological activities. In particular, it has the ability to stimulate IFN-γ production which augments NK activity in spleen cells. See Okamura, *et al.* (1995) Nature 378:88-91.

The activities of the mouse IL-1α, IL-1β, and IGIF have been compared as to their ability to induce IFN-γ, alone or in combination with IL-2 or IL-12 in SCID splenocytes and purified NK cells. See Hunter, *et al.* (1995) J. Immunol. 155:4347-4354; and Bancroft, *et al.* (1991) Immunol. Revs. 124:5-xxx. The IGIF was found to be much more potent in stimulating IFN-1γ than IL-1α or IL-1β.

In IL-2 activated NK cells, IFN-γ production is blocked by the addition of anti-IL-1β antibodies. See Hunter, *et al.* (1995). However, mouse IGIF can overcome this block and induce IFN-γ. This is the only cytokine known to be able to do this. In addition, *in vivo*, administration of mouse IGIF to mice infected with the parasite T. Cruzi significantly decreases parasitemia.

The present disclosure also describes new activities which have been discovered using the mouse IGIF molecule. Applicants have confirmed that the mouse IGIF molecule produced by similar recombinant means to the human IL-1γ protein characterized herein, exhibit the biological activity of inducing T cells to produce IFN-γ. See assays described, e.g., in de Waal Malefyt, *et al.*, in de Vries and de Waal Malefyt (eds. 1995) "Interleukin-10" Landes Co., Austin, TX. It also modestly stimulates IFN-γ by NK cells. But, as with the human described above, there is substantial synergy with IL-12.

Cross-species biological activity has not yet been detected with these assays, e.g., the biological activity of mouse IGIF on human cells has not been yet demonstrated, nor has the activity of human IL-1γ been demonstrated on mouse cells. This suggests that the receptors, which are expected to include multiple different polypeptide chains, exhibit species specificity for their corresponding ligands. The IL-1α and IL-1β ligands both signal through heterodimeric receptors.

This invention further contemplates use of isolated nucleic acid or fragments, e.g., which encode this or a closely related protein, or fragments thereof, to encode a biologically active corresponding polypeptide. In addition, this invention covers isolated or recombinant DNA which encodes a biologically active protein or polypeptide having characteristic IL-1γ activity. Typically, the nucleic acid is capable of hybridizing, under appropriate conditions, with a nucleic acid sequence segment of SEQ ID NO: 1. Said biologically active protein or polypeptide can be a full length protein, or fragment, and will typically have a segment

of amino acid sequence highly homologous to one shown in SEQ ID NO: 2. Further, this invention covers the use of isolated or recombinant nucleic acid, or fragments thereof, which encode proteins having fragments which are homologous to the disclosed IL-1γ protein. The isolated nucleic acids can have the respective regulatory sequences in the 5' and 3' flanks, e.g., promoters, enhancers, poly-A addition signals, and others from the natural gene.

An "isolated" nucleic acid is defined herein to mean a nucleic acid, e.g., an RNA, DNA, or a mixed polymer, which is substantially pure, e.g., separated from other components which naturally accompany a native sequence, such as ribosomes, polymerases, and flanking genomic sequences from the originating species. The term embraces a nucleic acid sequence which has been removed from its naturally occurring environment, and includes recombinant or cloned DNA isolates, which are thereby distinguishable from naturally occurring compositions, and chemically synthesized analogs or analogs biologically synthesized by heterologous systems. A substantially pure molecule includes isolated forms of the molecule, either completely or substantially pure.

An isolated nucleic acid will generally be a homogeneous composition of molecules but will in some embodiments contain heterogeneity, preferably minor. This heterogeneity is typically found at the polymer ends or portions not critical to a desired biological function or activity.

A "recombinant" nucleic acid is defined herein either by its method of production or its structure. In reference to its method of production, e.g., a product made by a process, the process is use of recombinant nucleic acid techniques, e.g., involving human intervention in the nucleotide sequence. Typically this intervention involves *in vitro* manipulation, although under certain circumstances it may involve more classical animal breeding techniques. Alternatively, it can be a nucleic acid made by generating a sequence comprising

fusion of two fragments which are not naturally contiguous to each other, but is meant to exclude products of nature, e.g., naturally occurring mutants as found in their natural state. Thus, for example, products made by transforming cells with any unnaturally occurring vector is encompassed, as are nucleic acids comprising sequence derived using any synthetic oligonucleotide process. Such a process is often done to replace a codon with a redundant codon encoding the same or a conservative amino acid, while typically introducing or removing a restriction enzyme sequence recognition site.

Alternatively, the process is performed to join together nucleic acid segments of desired functions to generate a single genetic entity comprising a desired combination of functions not found in the commonly available natural forms, e.g., encoding a fusion protein. Restriction enzyme recognition sites are often the target of such artificial manipulations, but other site specific targets, e.g., promoters, DNA replication sites, regulation sequences, control sequences, or other useful features may be incorporated by design. A similar concept is intended for a recombinant, e.g., fusion, polypeptide. This will include a dimeric repeat. Specifically included are synthetic nucleic acids which, by genetic code redundancy, encode similar polypeptides to fragments of the IL-1γ, and fusions of sequences from various different interleukin or related molecules, e.g., growth factors.

A "fragment" in a nucleic acid context is defined herein to mean a contiguous segment of at least about 17 nucleotides, generally at least 21 nucleotides, more generally at least 25 nucleotides, ordinarily at least 30 nucleotides, more ordinarily at least 35 nucleotides, often at least 39 nucleotides, more often at least 45 nucleotides, typically at least 50 nucleotides, more typically at least 55 nucleotides, usually at least 60 nucleotides, more usually at least 66 nucleotides, preferably at least 72 nucleotides, more preferably at

least 79 nucleotides, and in particularly preferred embodiments will be at least 85 or more nucleotides. Typically, fragments of different genetic sequences can be compared to one another over appropriate length stretches.

A nucleic acid which codes for an IL-1γ will be particularly useful to identify genes, mRNA, and cDNA species which code for itself or closely related proteins, as well as DNAs which code for polymorphic, allelic, or other genetic variants, e.g., from different individuals. Preferred probes for such screens are those regions of the interleukin which are conserved between different polymorphic variants or which contain nucleotides which lack specificity, and will preferably be full length or nearly so. In other situations, polymorphic variant specific sequences will be more useful.

This invention further covers recombinant nucleic acid molecules and fragments having a nucleic acid sequence identical to or highly homologous to the isolated DNA set forth herein. In particular, the sequences will often be operably linked to DNA segments which control transcription, translation, and DNA replication. These additional segments typically assist in expression of the desired nucleic acid segment.

Homologous nucleic acid sequences, when compared to one another or SEQ ID NO: 1 sequences, exhibit significant similarity. The standards for homology in nucleic acids are either measures for homology generally used in the art by sequence comparison or based upon hybridization conditions. Comparative hybridization conditions are described in greater detail below.

Substantial homology in the nucleic acid sequence comparison context means either that the segments, or their complementary strands, when compared, are identical when optimally aligned, with appropriate nucleotide insertions or deletions, in at least about 60% of the nucleotides, generally at least 66%, ordinarily at least 71%, often at least 76%, more often at least 80%, usually

at least 84%, more usually at least 88%, typically at least 91%, more typically at least about 93%, preferably at least about 95%, more preferably at least about 96 to 98% or more, and in particular embodiments, as high at about 99% or more of the nucleotides.

Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions, to a strand or its complement, typically using a sequence derived from SEQ ID NO: 1. Typically, selective hybridization will occur when there is at least about 55% homology over a stretch of at least about 14 nucleotides, more typically at least about 65%, preferably at least about 75%, and more preferably at least about 90%. See, Kanehisa (1984) Nuc. Acids Res. 12:203-213, which is incorporated herein by reference. The length of homology comparison, as described, may be over longer stretches, and in certain embodiments will be over a stretch of at least about 17 nucleotides, generally at least about 20 nucleotides, ordinarily at least about 24 nucleotides, usually at least about 28 nucleotides, typically at least about 32 nucleotides, more typically at least about 40 nucleotides, preferably at least about 50 nucleotides, and more preferably at least about 75 to 100 or more nucleotides. Preferably a related nucleic acid will contain a plurality of such matching fragments, e.g., at elast 2, preferably 3, 4, 5, or more of specific, or assorted, lengths.

Stringent conditions, in referring to homology in the hybridization context, will be stringent combined conditions of salt, temperature, organic solvents, and other parameters typically controlled in hybridization reactions. Stringent temperature conditions will usually include temperatures in excess of about 30° C, more usually in excess of about 37° C, typically in excess of about 45° C, more typically in excess of about 55° C, preferably in excess of about 65° C, and more preferably in excess of about 70° C. Stringent salt conditions will ordinarily be less than about 500 mM, usually less than about 400 mM, more usually less than about 300 mM,

typically less than about 200 mM, preferably less than about 100 mM, and more preferably less than about 80 mM, even down to less than about 20 mM. However, the combination of parameters is much more important than the measure of any single parameter. See, e.g., Wetmur and Davidson (1968) J. Mol. Biol. 31:349-370.

The isolated DNA can be readily modified by nucleotide substitutions, nucleotide deletions, nucleotide insertions, and inversions of nucleotide stretches. These modifications result in novel DNA sequences which encode this protein, its derivatives, or proteins having IL-1γ activity. These modified sequences can be used to produce mutant proteins (muteins) or to enhance the expression of variant species. Enhanced expression may involve gene amplification, increased transcription, increased translation, and other mechanisms. Such mutant IL-1γ derivatives include predetermined or site-specific mutations of the protein or its fragments, including silent mutations using genetic code degeneracy.

"Mutant IL-1γ" as defined herein encompasses a polypeptide otherwise falling within the homology definition of the human IL-1γ as set forth above, but having an amino acid sequence which differs from that of human IL-1γ as found in nature, whether by way of deletion, substitution, or insertion. In particular, "site specific mutant IL-1γ" encompasses a protein having substantial homology with the sequence of SEQ ID NO: 2, and typically shares most of the biological activities of the form disclosed herein.

Although site specific mutation sites are predetermined, mutants need not be site specific. Human IL-1γ mutagenesis can be achieved by making amino acid insertions or deletions in the gene, coupled with expression. Substitutions, deletions, insertions, or any combinations may be generated to arrive at a final construct. Insertions include amino- or carboxy-terminal fusions. Random mutagenesis can be conducted at a target codon and the expressed human IL-1γ

mutants can then be screened for the desired activity. Methods for making substitution mutations at predetermined sites in DNA having a known sequence are well known in the art, e.g., by M13 primer mutagenesis. See also Sambrook, *et al.* (1989) and Ausubel, *et al.* (1987 and periodic Supplements).

The mutations in the DNA normally should not place coding sequences out of reading frames and preferably will not create complementary regions that could hybridize to produce secondary mRNA structure such as loops or hairpins.

The phosphoramidite method described by Beaucage and Carruthers (1981) Tetra. Letts. 22:1859-1862, will produce suitable synthetic DNA fragments. A double stranded fragment will often be obtained either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Polymerase chain reaction (PCR) techniques can often be applied in mutagenesis. Alternatively, mutagenisis primers are commonly used methods for generating defined mutations at predetermined sites. See, e.g, Innis, *et al.* (eds. 1990) PCR Protocols: A Guide to Methods and Applications Academic Press, San Diego, CA.

As described above, the present invention encompasses the human IL-1γ whose sequence is defined in SEQ ID NO: 2 and described above. Allelic and other variants are also contemplated.

The present invention also provides recombinant proteins, e.g., heterologous fusion proteins using segments from this human protein. A heterologous fusion protein is a fusion of proteins or segments which are naturally not normally fused in the same manner. Thus, the fusion product of a growth factor with an interleukin is a continuous protein molecule having sequences fused in a typical peptide linkage, typically made as a single translation product and exhibiting properties derived

from each source peptide. A similar concept applies to heterologous nucleic acid sequences.

In addition, new constructs may be made from combining similar functional or structural domains from other related proteins, e.g., growth factors or other cytokines. For example, receptor-binding or other segments may be "swapped" between different new fusion polypeptides or fragments. See, e.g., Cunningham, *et al.* (1989) Science 243:1330-1336; and O'Dowd, *et al.* (1988) J. Biol. Chem. 263:15985-15992, each of which is incorporated herein by reference. Thus, new chimeric polypeptides exhibiting new combinations of specificities will result from the functional linkage of receptor binding specificities. For example, the receptor binding domains from other related ligand molecules may be added or substituted for other domains of this or related proteins. The resulting protein will often have hybrid function and properties.

For example, a fusion protein may include a targetting domain which may serve to provide sequestering of the fusion protein to a particular organ, e.g., a ligand portions which is specifically bound by spleen cells and would serve to accumulate in the spleen.

Candidate fusion partners and sequences can be selected from various sequence data bases, e.g., GenBank, c/o IntelliGenetics, Mountain View, CA; and BCG, University of Wisconsin Biotechnology Computing Group, Madison, WI.

The present invention particularly provides muteins which act as agonists or antagonists of the IL-1γ. Structural alignment of human and mouse IL1γ with other members of IL-1 family show conserved features/residues, particularly 12 β strands folded into β-trefoil fold. See Bazan, *et al.* (1996) Nature 379:591. Alignment with the human IL-1γ sequence (using the met initiation residue of the signal peptide) indicates that the β1 (leu 41 to val 47), β2 (val 55 to asp 59), β3 (pro 64 to asp 68), β4 (phe 83 to tyr 88), β5 (met 96 to val 102), β6 (ser 108 to glu 113), β7 (lys 115 to lys 120), β8 (phe

137 to pro 143), β9 (asn 147 to ser 153), β10 (phe 160 to glu 164), β11 (phe 170 to lys 175), and β12 (phe 187 to asn 191) strands correspond to similar sequence in the mouse IGIF. See also, Lodi, *et al.* (1994) Science 263:1762-1766; Sayle and Milner-White (1995) TIBS 20:374-376; and Gronenberg, *et al.* (1991) Protein Engineering 4:263-269.

The IL-1α and IL-1β ligands bind an IL-1 receptor type I as the primary receptor and this complex then forms a high affinity receptor complex with the IL-1 receptor type III. The mouse IL-1γ does not bind to the known mouse IL-1 receptor types I, II (decoy receptor), or III. In addition, the mouse IGIF biological activity cannot be blocked with anti-type I, II, or III antibodies. This suggests that the related mouse IGIF binds to receptors related to the IL-1 receptors already isolated, but not yet identified as receptors for the IGIF.

However, the solved structures for IL-1β, the natural IL-1 receptor antagonist (IL-1Ra), and a co-structure of IL-1Ra/IL-1 receptor type I, suggest how to make a mouse IGIF or a human IL-1γ antagonist. The loop between the β4 strand and the β5 strand is the primary binding segment for other IL-1 ligands to the receptor type III. In IL-1α and IL-1β ligands, this loop spans 8 residues, while in IL-1Ra, this loop is "cut off" (only 2 residues remain). Therefore, IL-1Ra binds normally to receptor type I, but can not interact with receptor type III. This makes IL-1Ra into an effective IL-1 antagonist.

This suggests that modifications to the loop between the β4 and the β5 strands will lead to variants with predictable biological activities. For example, substitution of KDSEVRGL (residues between β4 and β5 strand, with the first residue in the β5 strand) in the mouse IGIF (or the corresponding KDSQPRGM of human IL-1γ to EPH) with EPH (corresponding IL-1Ra residues) should generate an IGIF antagonist. Alternatively, substitution of KDSEVR of mouse IGIF with QGEESND (IL-1β residues

between β4 and β5 strand) should allow interaction with the type III receptor (in human IL-1γ, substitute KDSQPR with QGEESND). With mouse IL-1Ra, it was shown that replacement of the mouse IL-1Ra residues with these mouse IL-1β residues introduced IL-1 activity in IL-1Ra (IL-1Ra can now bind type III receptor). This will establish whether type III receptor can be used by mouse IGIF.

In addition, a construct which substitutes KDSEVR of the mouse IGIF with the Flag epitope DYKDDDDK would probably bind to the primary IGIF receptor. In human IL-1γ, the substitution would be KDSQPR with DYKDDDDK. This mutant protein could act as an antagonist (unable to bind secondary mIL-1γ receptor). This tagged molecule should be useful to clone the mouse IGIF primary receptor, which has not been identified as such.

Similar variations in the human IL-1γ ligand sequence, e.g., in the corresponding region between residues 88-96, should provide similar interactions with receptor. Substitutions with either mouse sequences or human sequences are indicated. Conversely, conservative substitutions away from the receptor binding interaction regions will probably preserve most biological activities.

"Derivatives" of the human IL-1γ include amino acid sequence mutants, glycosylation variants, metabolic derivatives and covalent or aggregative conjugates with other chemical moieties. Covalent derivatives can be prepared by linkage of functionalities to groups which are found in the IL-1γ amino acid side chains or at the N- or C-termini, e.g., by means which are well known in the art. These derivatives can include, without limitation, aliphatic esters or amides of the carboxyl terminus, or of residues containing carboxyl side chains, O-acyl derivatives of hydroxyl group-containing residues, and N-acyl derivatives of the amino terminal amino acid or amino-group containing residues, e.g., lysine or arginine. Acyl groups are selected from the group of alkyl-moieties including C3 to C18 normal alkyl, thereby forming alkanoyl aroyl species.

In particular, glycosylation alterations are included, e.g., made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing, or in further processing steps. Particularly preferred means for accomplishing this are by exposing the polypeptide to glycosylating enzymes derived from cells which normally provide such processing, e.g., mammalian glycosylation enzymes. Deglycosylation enzymes are also contemplated. Also embraced are versions of the same primary amino acid sequence which have other minor modifications, including phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

A major group of derivatives are covalent conjugates of the interleukin or fragments thereof with other proteins of polypeptides. These derivatives can be synthesized in recombinant culture such as N- or C-terminal fusions or by the use of agents known in the art for their usefulness in cross-linking proteins through reactive side groups. Preferred derivatization sites with cross-linking agents are at free amino groups, carbohydrate moieties, and cysteine residues.

Fusion polypeptides between the interleukin and other homologous or heterologous proteins are also provided. Homologous polypeptides may be fusions between different growth factors, resulting in, for instance, a hybrid protein exhibiting ligand specificity for multiple different receptors, or a ligand which may have broadened or weakened specificity of binding to its receptor. Likewise, heterologous fusions may be constructed which would exhibit a combination of properties or activities of the derivative proteins. Typical examples are fusions of a reporter polypeptide, e.g., luciferase, with a segment or domain of a receptor, e.g., a ligand-binding segment, so that the presence or location of a desired ligand may be easily determined. See, e.g., Dull, *et al.*, U.S. Patent No. 4,859,609. Other gene fusion partners include glutathione-S-transferase (GST), bacterial ß-galactosidase, trpE, Protein A, ß-lactamase,

alpha amylase, alcohol dehydrogenase, and yeast alpha mating factor. See, e.g., Godowski, *et al.* (1988) Science 241:812-816.

The phosphoramidite method described by Beaucage and Carruthers (1981) Tetra. Letts. 22:1859-1862, will produce suitable synthetic DNA fragments. A double stranded fragment will often be obtained either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Such polypeptides may also have amino acid residues which have been chemically modified by phosphorylation, sulfonation, biotinylation, or the addition or removal of other moieties, particularly those which have molecular shapes similar to phosphate groups. In some embodiments, the modifications will be useful labeling reagents, or serve as purification targets, e.g., affinity ligands.

Fusion proteins will typically be made by either recombinant nucleic acid methods or by synthetic polypeptide methods. Techniques for nucleic acid manipulation and expression are described generally, for example, in Sambrook, *et al.* (1989) Molecular Cloning: A Laboratory Manual (2d ed.), Vols. 1-3, Cold Spring Harbor Laboratory, and Ausubel, *et al.* (eds)(1987 and periodic supplements) Current Protocols in Molecular Biology, Greene/Wiley, New York, which are each incorporated herein by reference. Techniques for synthesis of polypeptides are described, for example, in Merrifield (1963) J. Amer. Chem. Soc. 85:2149-2156; Merrifield (1986) Science 232: 341-347; and Atherton, *et al.* (1989) Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, Oxford; each of which is incorporated herein by reference. See also Dawson, *et al.* (1994) Science 266:776-779 for methods to make larger polypeptides.

This invention also contemplates the use of derivatives of the human IL-1γ other than variations in amino acid sequence or glycosylation. Such derivatives may involve covalent or aggregative association with chemical moieties. These derivatives generally fall into three classes: (1) salts, (2) side chain and terminal residue covalent modifications, and (3) adsorption complexes, for example with cell membranes. Such covalent or aggregative derivatives are useful as immunogens, as reagents in immunoassays, or in purification methods such as for affinity purification of a receptor or other binding molecule, e.g., an antibody.

For example, the human IL-1γ ligand can be immobilized by covalent bonding to a solid support such as cyanogen bromide-activated Sepharose, by methods which are well known in the art, or adsorbed onto polyolefin surfaces, with or without glutaraldehyde cross-linking, for use in the assay or purification of IL-1γ receptor, antibodies, or other similar molecules. The IL-1γ can also be labeled with a detectable group, for example radioiodinated by the chloramine T procedure, covalently bound to rare earth chelates, or conjugated to another fluorescent moiety for use in diagnostic assays.

The human IL-1γ of this invention can be used as an immunogen for the production of antisera or antibodies specific, e.g., capable of distinguishing between mouse IGIF and human IL-1γ, for the interleukin or any fragments thereof. The purified interleukin can be used to screen monoclonal antibodies or antigen-binding fragments prepared by immunization with various forms of impure preparations containing the protein. In particular, the term "antibodies" also encompasses antigen binding fragments of natural antibodies. The purified interleukin can also be used as a reagent to detect any antibodies generated in response to the presence of elevated levels of expression, or immunological disorders which lead to antibody production to the endogenous cytokine. Additionally, IL-1γ fragments may also serve as immunogens to produce the

antibodies of the present invention, as described immediately below. For example, this invention contemplates antibodies having binding affinity to or being raised against the amino acid sequence shown in SEQ ID NO: 2, fragments thereof, or homologus peptides. In particular, this invention contemplates antibodies having binding affinity to, or having been raised against, specific fragments which are predicted to be, or actually are, exposed at the exterior protein surface of the native cytokine.

The blocking of physiological response to these interleukins may result from the inhibition of binding of the ligand to the receptor, likely through competitive inhibition. Thus, *in vitro* assays of the present invention will often use antibodies or ligand binding segments of these antibodies, or fragments attached to solid phase substrates. These assays will also allow for the diagnostic determination of the effects of either binding region mutations and modifications, or ligand mutations and modifications, e.g., ligand analogs.

This invention also contemplates the use of competitive drug screening assays, e.g., where neutralizing antibodies to the interleukin or fragments compete with a test compound for binding to a receptor or antibody. In this manner, the neutralizing antibodies or fragments can be used to detect the presence of any polypeptide which shares one or more binding sites to a receptor and can also be used to occupy binding sites on a receptor that might otherwise bind an interleukin.

DNA which encodes the protein or fragments thereof can be obtained by chemical synthesis, screening cDNA libraries, or by screening genomic libraries prepared from a wide variety of cell lines or tissue samples. Natural sequences can be isolated using standard methods and the sequences provided herein in SEQ ID NO: 1.

This DNA can be expressed in a wide variety of host cells for the synthesis of a full-length human interleukin or fragments which can in turn, for example, be used to generate polyclonal or monoclonal antibodies;

for binding studies; for construction and expression of modified agonist/antagonist molecules; and for structure/function studies. Each variant or its fragments can be expressed in host cells that are transformed or transfected with appropriate expression vectors. These molecules can be substantially free of protein or cellular contaminants, other than those derived from the recombinant host, and therefore are particularly useful in pharmaceutical compositions when combined with a pharmaceutically acceptable carrier and/or diluent. The human protein, or portions thereof, may be expressed as fusions with other proteins.

Expression vectors are typically self-replicating DNA or RNA constructs containing the desired receptor gene or its fragments, usually operably linked to suitable genetic control elements that are recognized in a suitable host cell. These control elements are capable of effecting expression within a suitable host. The specific type of control elements necessary to effect expression will depend upon the eventual host cell used. Generally, the genetic control elements can include a prokaryotic promoter system or a eukaryotic promoter expression control system, and typically include a transcriptional promoter, an optional operator to control the onset of transcription, transcription enhancers to elevate the level of mRNA expression, a sequence that encodes a suitable ribosome binding site, and sequences that terminate transcription and translation. Expression vectors also usually contain an origin of replication that allows the vector to replicate independently of the host cell.

The vectors of this invention include those which contain DNA which encodes a protein, as described, or a fragment thereof encoding a biologically active equivalent polypeptide. The DNA can be under the control of a viral promoter and can encode a selection marker. This invention further contemplates use of such expression vectors which are capable of expressing eukaryotic cDNA coding for such a protein in a

prokaryotic or eukaryotic host, where the vector is compatible with the host and where the eukaryotic cDNA coding for the receptor is inserted into the vector such that growth of the host containing the vector expresses the cDNA in question. Usually, expression vectors are designed for stable replication in their host cells or for amplification to greatly increase the total number of copies of the desirable gene per cell. It is not always necessary to require that an expression vector replicate in a host cell, e.g., it is possible to effect transient expression of the interleukin protein or its fragments in various hosts using vectors that do not contain a replication origin that is recognized by the host cell. It is also possible to use vectors that cause integration of the human protein or its fragments into the host DNA by recombination.

Vectors, as used herein, comprise plasmids, viruses, bacteriophage, integratable DNA fragments, and other vehicles which enable the integration of DNA fragments into the genome of the host. Expression vectors are specialized vectors which contain genetic control elements that effect expression of operably linked genes. Plasmids are the most commonly used form of vector but all other forms of vectors which serve an equivalent function and which are, or become, known in the art are suitable for use herein. See, e.g., Pouwels, *et al.* (1985 and Supplements) Cloning Vectors: A Laboratory Manual, Elsevier, N.Y., and Rodriquez, *et al.* (eds) Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Buttersworth, Boston, 1988, which are incorporated herein by reference.

Transformed cells are cells, preferably mammalian, that have been transformed or transfected with receptor vectors constructed using recombinant DNA techniques. Transformed host cells usually express the desired protein or its fragments, but for purposes of cloning, amplifying, and manipulating its DNA, do not need to express the subject protein. This invention further contemplates culturing transformed cells in a nutrient

medium, thus permitting the interleukin to accumulate in the culture. The protein can be recovered, either from the culture or from the culture medium.

For purposes of this invention, nucleic sequences are operably linked when they are functionally related to each other. For example, DNA for a presequence or secretory leader is operably linked to a polypeptide if it is expressed as a preprotein or participates in directing the polypeptide to the cell membrane or in secretion of the polypeptide. A promoter is operably linked to a coding sequence if it controls the transcription of the polypeptide; a ribosome binding site is operably linked to a coding sequence if it is positioned to permit translation. Usually, operably linked means contiguous and in reading frame, however, certain genetic elements such as repressor genes are not contiguously linked but still bind to operator sequences that in turn control expression.

Suitable host cells include prokaryotes, lower eukaryotes, and higher eukaryotes. Prokaryotes include both gram negative and gram positive organisms, e.g., *E. coli* and *B. subtilis*. Lower eukaryotes include yeasts, e.g., *S. cerevisiae* and *Pichia*, and species of the genus *Dictyostelium*. Higher eukaryotes include established tissue culture cell lines from animal cells, both of non-mammalian origin, e.g., insect cells, and birds, and of mammalian origin, e.g., human, primates, and rodents.

Prokaryotic host-vector systems include a wide variety of vectors for many different species. As used herein, *E. coli* and its vectors will be used generically to include equivalent vectors used in other prokaryotes. A representative vector for amplifying DNA is pBR322 or many of its derivatives. Vectors that can be used to express the receptor or its fragments include, but are not limited to, such vectors as those containing the lac promoter (pUC-series); trp promoter (pBR322-trp); Ipp promoter (the pIN-series); lambda-pP or pR promoters (pOTS); or hybrid promoters such as ptac (pDR540). See

Brosius, *et al.* (1988) "Expression Vectors Employing Lambda-, trp-, lac-, and Ipp-derived Promoters", in Vectors: A Survey of Molecular Cloning Vectors and Their Uses, (eds. Rodriguez and Denhardt), Buttersworth, Boston, Chapter 10, pp. 205-236, which is incorporated herein by reference.

Lower eukaryotes, e.g., yeasts and *Dictyostelium*, may be transformed with IL-1γ sequence containing vectors. For purposes of this invention, the most common lower eukaryotic host is the baker's yeast, *Saccharomyces cerevisiae*. It will be used to generically represent lower eukaryotes although a number of other strains and species are also available. Yeast vectors typically consist of a replication origin (unless of the integrating type), a selection gene, a promoter, DNA encoding the receptor or its fragments, and sequences for translation termination, polyadenylation, and transcription termination. Suitable expression vectors for yeast include such constitutive promoters as 3-phosphoglycerate kinase and various other glycolytic enzyme gene promoters or such inducible promoters as the alcohol dehydrogenase 2 promoter or metallothionine promoter. Suitable vectors include derivatives of the following types: self-replicating low copy number (such as the YRp-series), self-replicating high copy number (such as the YEp-series); integrating types (such as the YIp-series), or mini-chromosomes (such as the YCp-series).

Higher eukaryotic tissue culture cells are normally the preferred host cells for expression of the functionally active interleukin protein. In principle, any higher eukaryotic tissue culture cell line is workable, e.g., insect baculovirus expression systems, whether from an invertebrate or vertebrate source. However, mammalian cells are preferred. Transformation or transfection and propagation of such cells has become a routine procedure. Examples of useful cell lines include HeLa cells, Chinese hamster ovary (CHO) cell lines, baby rat kidney (BRK) cell lines, insect cell

lines, bird cell lines, and monkey (COS) cell lines. Expression vectors for such cell lines usually include an origin of replication, a promoter, a translation initiation site, RNA splice sites (if genomic DNA is used), a polyadenylation site, and a transcription termination site. These vectors also usually contain a selection gene or amplification gene. Suitable expression vectors may be plasmids, viruses, or retroviruses carrying promoters derived, e.g., from such sources as from adenovirus, SV40, parvoviruses, vaccinia virus, or cytomegalovirus. Representative examples of suitable expression vectors include pCDNA1; pCD, see Okayama, *et al.* (1985) Mol. Cell Biol. 5:1136-1142; pMC1neo PolyA, see Thomas, *et al.* (1987) Cell 51:503-512; and a baculovirus vector such as pAC 373 or pAC 610.

For secreted proteins, an open reading frame usually encodes a polypeptide that consists of a mature or secreted product covalently linked at ist N-terminus to a signal peptide. The signal peptide is cleaved prior to secretion of the mature, or active, polypeptide. The cleavage site can be predicted with a high degree of accuracy from empirical rules, e.g., von-Heijne (1986) Nucleic Acids Research 14:4683-4690, and the precise amino acid composition of the signal peptide does not appear to be critical to its function, e.g., Randall, *et al.* (1989) Science 243:1156-1159; Kaiser st al. (1987) Science 235:312-317.

It will often be desired to express these polypeptides in a system which provides a specific or defined glycosylation pattern. In this case, the usual pattern will be that provided naturally by the expression system. However, the pattern will be modifiable by exposing the polypeptide, e.g., an unglycosylated form, to appropriate glycosylating proteins introduced into a heterologous expression system. For example, the interleukin gene may be co-transformed with one or more genes encoding mammalian or other glycosylating enzymes. Using this approach, certain mammalian glycosylation patterns will be achievable in prokaryote or other cells.

The source of human IL-1γ can be a eukaryotic or prokaryotic host expressing recombinant huIL-1γ DNA, such as is described above. The source can also be a cell line such as mouse Swiss 3T3 fibroblasts, but other mammalian cell lines are also contemplated by this invention, with the preferred cell line being from the human species.

Now that the entire sequence is known, the human IL-1γ, fragments, or derivatives thereof can be prepared by conventional processes for synthesizing peptides. These include processes such as are described in Stewart and Young (1984) Solid Phase Peptide Synthesis, Pierce Chemical Co., Rockford, IL; Bodanszky and Bodanszky (1984) The Practice of Peptide Synthesis, Springer-Verlag, New York; and Bodanszky (1984) The Principles of Peptide Synthesis, Springer-Verlag, New York; all of each which are incorporated herein by reference. For example, an azide process, an acid chloride process, an acid anhydride process, a mixed anhydride process, an active ester process (for example, p-nitrophenyl ester, N-hydroxysuccinimide ester, or cyanomethyl ester), a carbodiimidazole process, an oxidative-reductive process, or a dicyclohexylcarbodiimide (DCCD)/additive process can be used. Solid phase and solution phase syntheses are both applicable to the foregoing processes.

The IL-1γ protein, fragments, or derivatives are suitably prepared in accordance with the above processes as typically employed in peptide synthesis, generally either by a so-called stepwise process which comprises condensing an amino acid to the terminal amino acid, one by one in sequence, or by coupling peptide fragments to the terminal amino acid. Amino groups that are not being used in the coupling reaction typically must be protected to prevent coupling at an incorrect location.

If a solid phase synthesis is adopted, the C-terminal amino acid is bound to an insoluble carrier or support through its carboxyl group. The insoluble carrier is not particularly limited as long as it

has a binding capability to a reactive carboxyl group. Examples of such insoluble carriers include halomethyl resins, such as chloromethyl resin or bromomethyl resin, hydroxymethyl resins, phenol resins, tert-alkyloxycarbonylhydrazidated resins, and the like.

An amino group-protected amino acid is bound in sequence through condensation of its activated carboxyl group and the reactive amino group of the previously formed peptide or chain, to synthesize the peptide step by step. After synthesizing the complete sequence, the peptide is split off from the insoluble carrier to produce the peptide. This solid-phase approach is generally described by Merrifield, *et al.* (1963) in J. Am. Chem. Soc. 85:2149-2156, which is incorporated herein by reference.

The prepared protein and fragments thereof can be isolated and purified from the reaction mixture by means of peptide separation, for example, by extraction, precipitation, electrophoresis, various forms of chromatography, and the like. The interleukin of this invention can be obtained in varying degrees of purity depending upon its desired use. Purification can be accomplished by use of the protein purification techniques disclosed herein, see below, or by the use of the antibodies herein described in methods of immunoabsorbant affinity chromatography. This immunoabsorbant affinity chromatography is carried out by first linking the antibodies to a solid support and then contacting the linked antibodies with solubilized lysates of appropriate cells, lysates of other cells expressing the interleukin, or lysates or supernatants of cells producing the protein as a result of DNA techniques, see below.

Generally, the purified protein will be at least about 40% pure, ordinarily at least about 50% pure, usually at least about 60% pure, typically at least about 70% pure, more typically at least about 80% pure, preferable at least about 90% pure and more preferably at least about 95% pure, and in particular embodiments,

97%-99% or more. Purity will usually be on a weight basis, but can also be on a molar basis. Different assays will be applied as appropriate.

Antibodies can be raised to the various human IL-1γ proteins and fragments thereof, both in naturally occurring native forms and in their recombinant forms, the difference being that antibodies to the active ligand are more likely to recognize epitopes which are only present in the native conformations.

Anti-idiotypic antibodies are also contemplated, which would be useful as agonists or antagonists of a natural receptor or an antibody.

Antibodies, including binding fragments and single chain versions, against predetermined fragments of the protein can be raised by immunization of animals with conjugates of the fragments with immunogenic proteins. Monoclonal antibodies are prepared from cells secreting the desired antibody. These antibodies can be screened for binding to normal or defective protein, or screened for agonistic or antagonistic activity. These monoclonal antibodies will usually bind with at least a K_D of about 1 mM, more usually at least about 300 µM, typically at least about 100µM, more typically at least about 30 µM, preferably at least about 10 µM, and more preferably at least about 3 µM or better.

The antibodies, including antibody antigen binding fragments, of this invention can have significant diagnostic or therapeutic value. They can be potent antagonists that bind to the interleukin and inhibit binding to the receptor or inhibit the ability of huamn IL-1γ to elicit a biological response. They also can be useful as non-neutralizing antibodies and can be coupled to toxins or radionuclides to bind producing cells, or cells localized to the source of the interleukin. Further, these antibodies can be conjugated to drugs or other therapeutic agents, either directly or indirectly by means of a linker.

As used in this invention, the term "antibody antigen binding fragment" includes, e.g., Fab, Fc, $F(ab)_2$, Fv and scFv fragments, which are employed with their standard immunological meanings. See, e.g., Klein, *Immunology* (John Wiley, New York, 1982); Parham, Chapter 14, *in* Weir, ed. *Immunochemistry*, 4th Ed. (Blackwell Scientific Publishers, Oxford, 1986). Such fragments can be made from intact antibodies by chemical cleavage of through the use of recombinant DNA methodology. See, e.g., U.S. Patent No. 4,642,334, describing the production of recombinant Fv fragments, and WO 93/11236 for scFv.

The antibodies of this invention can also be useful in diagnostic applications. As capture or non-neutralizing antibodies, they can bind to the interleukin without inhibiting receptor binding. As neutralizing antibodies, they can be useful in competitive binding assays. They will also be useful in detecting or quantifying IL-1γ.

Protein fragments may be joined to other materials, particularly polypeptides, as fused or covalently joined polypeptides to be used as immunogens. The human IL-1γ and its fragments may be fused or covalently linked to a variety of immunogens, such as keyhole limpet hemocyanin, bovine serum albumin, tetanus toxoid, etc. See Microbiology, Hoeber Medical Division, Harper and Row, 1969; Landsteiner (1962) Specificity of Serological Reactions, Dover Publications, New York; and Williams, *et al.* (1967) Methods in Immunology and Immunochemistry, Vol. 1, Academic Press, New York; each of which are incorporated herein by reference, for descriptions of methods of preparing polyclonal antisera. A typical method involves hyperimmunization of an animal with an antigen. The blood of the animal is then collected shortly after the repeated immunizations and the gamma globulin is isolated.

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, etc. Description of

techniques for preparing such monoclonal antibodies may be found in, e.g., Stites, *et al.* (eds) Basic and Clinical Immunology (4th ed.), Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane (1988) Antibodies: A Laboratory Manual, CSH Press; Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed) Academic Press, New York; and particularly in Kohler and Milstein (1975) in Nature 256: 495-497, which discusses one method of generating monoclonal antibodies. Each of these references is incorporated herein by reference. Summarized briefly, this method involves injecting an animal with an immunogen. The animal is then sacrificed and cells taken from its spleen, which are then fused with myeloma cells. The result is a hybrid cell or "hybridoma" that is capable of reproducing *in vitro*. The population of hybridomas is then screened to isolate individual clones, each of which secrete a single antibody species to the immunogen. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

Other suitable techniques involve *in vitro* exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors. See, Huse, *et al.* (1989) "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda," Science 246:1275-1281; and Ward, *et al.* (1989) Nature 341:544-546. The polypeptides and antibodies of the present invention may be used with or without modification, including chimeric or humanized antibodies. Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides,

enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents, teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant or chimeric immunoglobulins may be produced, see Cabilly, U.S. Patent No. 4,816,567.

The antibodies of this invention can also be used for affinity chromatography in isolating the IL-1γ. Columns can be prepared where the antibodies are linked to a solid support, e.g., particles, such as agarose, Sephadex, or the like, where a cell lysate may be passed through the column, the column washed, followed by increasing concentrations of a mild denaturant, whereby the purified protein will be released.

The antibodies may also be used to screen expression libraries for particular expression products. Usually the antibodies used in such a procedure will be labeled with a moiety allowing easy detection of presence of antigen by antibody binding.

Antibodies raised against each human IL-1γ will also be used to raise anti-idiotypic antibodies. These will be useful in detecting or diagnosing various immunological conditions related to expression of the protein or cells which express receptors for the protein. They also will be useful as agonists or antagonists of the interleukin, which may be competitive inhibitors or substitutes for naturally occurring ligands.

Both naturally occurring and recombinant forms of the human IL-1γ molecules of this invention are particularly useful in kits and assay methods. For example, these methods would also be applied to screening for binding activity, e.g., receptors for these proteins. Several methods of automating assays have been developed in recent years so as to permit screening of tens of thousands of compounds per year. See, e.g, a BIOMEK automated workstation, Beckman Instruments, Palo Alto, California, and Fodor, *et al.* (1991) Science 251:767-773, which is incorporated herein by reference. The latter

describes means for testing binding by a plurality of defined polymers synthesized on a solid substrate. The development of suitable assays to screen for a receptor or agonist/antagonist homologous proteins can be greatly facilitated by the availability of large amounts of purified, soluble IL-1γ in an active state such as is provided by this invention.

Purified IL-1γ can be coated directly onto plates for use in the aforementioned receptor screening techniques. However, non-neutralizing antibodies to these proteins can be used as capture antibodies to immobilize the respective interleukin on the solid phase, useful, e.g., in diagnostic uses.

This invention also contemplates use of IL-1γ, fragments thereof, peptides, and their fusion products in a variety of diagnostic kits and methods for detecting the presence of the protein or its receptor. Alternatively, or additionally, antibodies against the molecules may be incorporated into the kits and methods. Typically the kit will have a compartment containing either a defined IL-1γ peptide or gene segment or a reagent which recognizes one or the other. Typically, recognition reagents, in the case of peptide, would be a receptor or antibody, or in the case of a gene segment, would usually be a hybridization probe.

A preferred kit for determining the concentration of, for example, IL-1γ, a sample would typically comprise a labeled compound, e.g., receptor or antibody, having known binding affinity for IL-1γ, a source of IL-1γ (naturally occurring or recombinant) as a positive control, and a means for separating the bound from free labeled compound, for example a solid phase for immobilizing the IL-1γ in the test sample. Compartments containing reagents, and instructions, will normally be provided.

Antibodies, including antigen binding fragments, specific for human IL-1γ or a peptide fragment, or receptor fragments are useful in diagnostic applications to detect the presence of elevated levels of IL-1γ and/or

its fragments. Diagnostic assays may be homogeneous (without a separation step between free reagent and antibody-antigen complex) or heterogeneous (with a separation step). Various commercial assays exist, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay (EIA), enzyme-multiplied immunoassay technique (EMIT), substrate-labeled fluorescent immunoassay (SLFIA) and the like. For example, unlabeled antibodies can be employed by using a second antibody which is labeled and which recognizes the antibody to IL-1γ or to a particular fragment thereof. These assays have also been extensively discussed in the literature. See, e.g., Harlow and Lane (1988) Antibodies: A Laboratory Manual, CSH., and Coligan (Ed.) (1991) and periodic supplements, Current Protocols In Immunology Greene/Wiley, New York.

Anti-idiotypic antibodies may have similar use to serve as agonists or antagonists of IL-1γ. These should be useful as therapeutic reagents under appropriate circumstances.

Frequently, the reagents for diagnostic assays are supplied in kits, so as to optimize the sensitivity of the assay. For the subject invention, depending upon the nature of the assay, the protocol, and the label, either labeled or unlabeled antibody, or labeled receptor is provided. This is usually in conjunction with other additives, such as buffers, stabilizers, materials necessary for signal production such as substrates for enzymes, and the like. Preferably, the kit will also contain instructions for proper use and disposal of the contents after use. Typically the kit has compartments for each useful reagent, and will contain instructions for proper use and disposal of reagents. Desirably, the reagents are provided as a dry lyophilized powder, where the reagents may be reconstituted in an aqueous medium having appropriate concentrations for performing the assay.

Any of the aforementioned constituents of the diagnostic assays may be used without modification or may be modified in a variety of ways. For example, labeling may be achieved by covalently or non-covalently joining a moiety which directly or indirectly provides a detectable signal. In any of these assays, a test compound, IL-1γ, or antibodies thereto can be labeled either directly or indirectly. Possibilities for direct labeling include label groups: radiolabels such as ^{125}I, enzymes (U.S. Pat. No. 3,645,090) such as peroxidase and alkaline phosphatase, and fluorescent labels (U.S. Pat. No. 3,940,475) capable of monitoring the change in fluorescence intensity, wavelength shift, or fluorescence polarization. Both of the patents are incorporated herein by reference. Possibilities for indirect labeling include biotinylation of one constituent followed by binding to avidin coupled to one of the above label groups.

There are also numerous methods of separating the bound from the free ligand, or alternatively the bound from the free test compound. The IL-1γ can be immobilized on various matrixes followed by washing. Suitable matrices include plastic such as an ELISA plate, filters, and beads. Methods of immobilizing the receptor to a matrix include, without limitation, direct adhesion to plastic, use of a capture antibody, chemical coupling, and biotin-avidin. The last step in this approach involves the precipitation of antibody/antigen complex by any of several methods including those utilizing, e.g., an organic solvent such as polyethylene glycol or a salt such as ammonium sulfate. Other suitable separation techniques include, without limitation, the fluorescein antibody magnetizable particle method described in Rattle, *et al.* (1984) Clin. Chem. 30(9):1457-1461, and the double antibody magnetic particle separation as described in U.S. Pat. No. 4,659,678, each of which is incorporated herein by reference.

The methods for linking protein or fragments to various labels have been extensively reported in the literature and do not require detailed discussion here. Many of the techniques involve the use of activated carboxyl groups either through the use of carbodiimide or active esters to form peptide bonds, the formation of thioethers by reaction of a mercapto group with an activated halogen such as chloroacetyl, or an activated olefin such as maleimide, for linkage, or the like. Fusion proteins will also find use in these applications.

Another diagnostic aspect of this invention involves use of oligonucleotide or polynucleotide sequences taken from the sequence of an IL-1γ. These sequences can be used as probes for detecting levels of the IL-1γ in patients suspected of having an immulogoical disorder. The preparation of both RNA and DNA nucleotide sequences, the labeling of the sequences, and the preferred size of the sequences has received ample description and discussion in the literature. Normally an oligonucleotide probe should have at least about 14 nucleotides, usually at least about 18 nucleotides, and the polynucleotide probes may be up to several kilobases. Various labels may be employed, most commonly radionuclides, particularly ^{32}P. However, other techniques may also be employed, such as using biotin modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like.

Alternatively, antibodies may be employed which can recognize specific duplexes, including DNA duplexes, RNA duplexes, DNA-RNA hybrid duplexes, or DNA-protein duplexes. The antibodies in turn may be labeled and the assay carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected. The use of probes to the novel anti-sense RNA may be carried out in any conventional techniques such as

nucleic acid hybridization, plus and minus screening, recombinational probing, hybrid released translation (HRT), and hybrid arrested translation (HART). This also includes amplification techniques such as polymerase chain reaction (PCR).

Diagnostic kits which also test for the qualitative or quantitative presence of other markers are also contemplated. Diagnosis or prognosis may depend on the combination of multiple indications used as markers. Thus, kits may test for combinations of markers. See, e.g., Viallet, *et al.* (1989) Progress in Growth Factor Res. 1:89-97.

This invention also provides reagents with significant therapeutic value. The IL-1γ (naturally occurring or recombinant), fragments thereof, mutein agonists and antagonists, and antibodies, along with compounds identified as having binding affinity to the interleukin or its receptor or antibodies, should be useful in the treatment of conditions exhibiting abnormal expression of the interleukin. Such abnormality will typically be manifested by immunological disorders. Additionally, this invention should provide therapeutic value in various diseases or disorders associated with abnormal expression or abnormal triggering of response to the interleukin. The mouse IGIF has been suggested to be involved in tumors, allergies, and infectious diseases, e.g., pulmonary tuberculosis, leprosy, fulminant hepatitis, and viral infections, such as HIV.

In addition, the dendritic cell expression profile shows human IL-1γ primarily expressed in activated dendritic cells. Activated dendritic cells are also a major producer of IL-12, and it is thought that this dendritic cell produced IL-12 plays a major role in directing a Th1 type response. The combination of IL-1γ and IL-12 should be extremely potent in inducing IFN-γ. It is possible that the combination of these two pro-inflammatory cytokines under certain circumstances could lead to septic shock. An IL-1γ antagonist, mutein or antibody, could prove very useful in this situation.

It is possible that a natural IL-1γ antagonist is produced by NK cells or activated T-cells to counteract the effects of IL-1γ.

Recombinant IL-1γ, mutein agonists or antagonists, or IL-1γ antibodies can be purified and then administered to a patient. These reagents can be combined for therapeutic use with additional active ingredients, e.g., in conventional pharmaceutically acceptable carriers or diluents, along with physiologically innocuous stabilizers and excipients. These combinations can be sterile filtered and placed into dosage forms as by lyophilization in dosage vials or storage in stabilized aqueous preparations. This invention also contemplates use of antibodies or binding fragments thereof which are not complement binding.

Receptor screening using IL-1γ or fragments thereof can be performed to identify molecules having binding affinity to the interleukin. Subsequent biological assays can then be utilized to determine if a receptor can provide competitive binding, which can block intrinsic stimulating activity. Receptor fragments can be used as a blocker or antagonist in that it blocks the activity of IL-1γ. Likewise, a compound having intrinsic stimulating activity can activate the receptor and is thus an agonist in that it simulates the activity of IL-1γ. This invention further contemplates the therapeutic use of antibodies to IL-1γ and other molecules as antagonists.

The quantities of reagents necessary for effective therapy will depend upon many different factors, including means of administration, target site, physiological state of the patient, and other medicants administered. Thus, treatment dosages should be titrated to optimize safety and efficacy. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for in situ administration of these reagents. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage.

Various considerations are described, e.g., in Gilman, *et al.* (eds) (1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, 17th ed. (1990), Mack Publishing Co., Easton, Penn. Methods for administration are discussed therein and below, e.g., for oral, intravenous, intraperitoneal, or intramuscular administration, transdermal diffusion, and others. Pharmaceutically acceptable carriers will include water, saline, buffers, and other compounds described, e.g., in the Merck Index, Merck & Co., Rahway, New Jersey. Because of the likely high affinity binding between an IL-1γ and its receptors, low dosages of these reagents would be initially expected to be effective. Thus, dosage ranges would ordinarily be expected to be in amounts lower than 1 mM concentrations, typically less than about 10 μM concentrations, usually less than about 100 nM, preferably less than about 10 pM (picomolar), and most preferably less than about 1 fM (femtomolar), with an appropriate carrier. Slow release formulations, or slow release apparatus will often be utilized for continuous administration.

IL-1γ fragments thereof, and antibodies or its fragments, antagonists, and agonists, may be administered directly to the host to be treated or, depending on the size of the compounds, it may be desirable to conjugate them to carrier proteins such as ovalbumin or serum albumin prior to their administration. Therapeutic formulations may be administered in any conventional dosage formulation. While it is possible for the active ingredient to be administered alone, it is preferable to present it as a pharmaceutical formulation. Formulations comprise at least one active ingredient, as defined above, together with one or more acceptable carriers thereof. Each carrier must be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the patient. Formulations include those suitable for oral, rectal, nasal, or parenteral (including

subcutaneous, intramuscular, intravenous and intradermal) administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. See, e.g., Gilman, *et al.* (eds) (1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, 17th ed. (1990), Mack Publishing Co., Easton, Penn.; Avis, *et al.* (eds.) (1993) Pharmaceutical Dosage Forms: Parenteral Medications Dekker, NY; Lieberman, *et al.* (eds.) (1990) Pharmaceutical Dosage Forms: Tablets Dekker, NY; and Lieberman, *et al.* (eds.) (1990) Pharmaceutical Dosage Forms: Disperse Systems Dekker, NY. The therapy of this invention may be combined with or used in association with other therapeutic agents.

The description of the IL-1γ ligand herein provides means to identify a receptor, as described above. Such receptor should bind specifically to the IL-1γ with reasonably high affinity. Various constructs are made available which allow either labeling of the IL-1γ to detect its receptor. For example, directly labeling IL-1γ, fusing onto it markers for secondary labeling, e.g., FLAG or other epitope tags, etc., will allow detection of receptor. This can be histological, as an affinity method for biochemical purification, or labeling or selection in an expression cloning approach. A two-hybrid selection system may also be applied making appropriate constructs with the available IL-1γ sequences. See, e.g., Fields and Song (1989) Nature 340:245-246.

The broad scope of this invention is best understood with reference to the following examples, which are not intended to limit the inventions to the specific embodiments.

EXAMPLES

I. General Methods

Some of the standard methods are described or referenced, e.g., in Maniatis, *et al.* (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor Press; Sambrook, *et al.* (1989) Molecular Cloning: A Laboratory Manual, (2d ed.), vols 1-3, CSH Press, NY; Ausubel, *et al.*, Biology, Greene Publishing Associates, Brooklyn, NY; or Ausubel, *et al.* (1987 and Supplements) Current Protocols in Molecular Biology, Greene/Wiley, New York. Methods for protein purification include such methods as ammonium sulfate precipitation, column chromatography, electrophoresis, centrifugation, crystallization, and others. See, e.g., Ausubel, *et al.* (1987 and periodic supplements); Deutscher (1990) "Guide to Protein Purification" in Methods in Enzymology, vol. 182, and other volumes in this series; and manufacturer's literature on use of protein purification products, e.g., Pharmacia, Piscataway, N.J., or Bio-Rad, Richmond, CA. Combination with recombinant techniques allow fusion to appropriate segments, e.g., to a FLAG sequence or an equivalent which can be fused via a protease-removable sequence. See, e.g., Hochuli (1989) Chemische Industrie 12:69-70; Hochuli (1990) "Purification of Recombinant Proteins with Metal Chelate Absorbent" in Setlow (ed.) Genetic Engineering, Principle and Methods 12:87-98, Plenum Press, N.Y.; and Crowe, *et al.* (1992) OIAexpress: The High Level Expression & Protein Purification System QUIAGEN, Inc., Chatsworth, CA.

Computer sequence analysis is performed, e.g., using available software programs, including those from the GCG (U. Wisconsin) and GenBank sources. Public sequence databases were also used, e.g., from GenBank and others.

Many techniques applicable to IL-4 and IL-10 may be applied to IL-1γ, as described, e.g., in U.S. Patent No. 5,017,691 (IL-4), U.S.S.N. 07/453,951 (IL-10), and U.S.S.N. 08/110,683 (IL-10 receptor).

II. Amplification of human IL-1γ fragment by PCR

Two primers are selected (see SEQ ID NO: 1). RT-PCR is used on an appropriate mRNA sample selected for the presence of message to produce a cDNA, e.g., an activated human monocyte cell sample.

III. Tissue distribution of human IL-1γ

Message for the gene encoding IL-1γ has been detected in dendritic cells. Expression is high in day 5 dendritic cells, LPS activated dendritic cells, GM-CSF and IL-4 treated dendritic cells, and in a mixture of dendritic cells. High expression has not been detected in B cells or NK cells. Low levels have been detected in some monocyte cell cDNA libraries. Expression is generally not detected in fetal tissue, though low levels have been seen in fetal spleen, lung, and small intestine. Low levels are also detected in fetal gall bladder, and adult tonsil.

IV. Production of mouse IGIF protein

A GST fusion construct was engineered for expression in *E. coli*. Protein was expressed, and purified using standard procedures. Similar methods are applicable to human IL-1γ.

A mouse IGIF pGex plasmid was constructed and transformed into *E. coli*. Freshly transformed cells were grown in LB medium containing 50 μg/ml ampicillin and induced with IPTG (Sigma, St. Louis, MO). After overnight induction, the bacteria were harvested and the pellets containing IGIF were isolated. The pellets were homogenized in TE buffer (50 mM Tris-base pH 8.0, 10 mM EDTA and 2 mM pefabloc) in 2 liters. This material was

passed through a microfluidizer (Microfluidics, Newton, MA) three times. The fluidized supernatant was spun down on a Sorvall Gs-3 rotor for 1 h at 13,000 rpm. The resulting supernatant containing the IGIF was filtered and passed over a glutathione-SEPHAROSE column equilibrated in 50 mM Tris-base pH 8.0. The fractions containing the IGIF-GST fusion protein were pooled and cleaved with thrombin (Enzyme Research Laboratories, Inc., South Bend, IN). The cleaved pool was then passed over a Q-SEPHAROSE column equilibrated in 50 mM Tris-base. Fractions containing IGIF were pooled and diluted in cold distilled H_2O, to lower the conductivity, and passed back over a fresh Q-Sepharose column. The fractions containing IGIF were pooled, aliquoted, and stored in the -70° C freezer.

Comparision of the CD spectrum with mouse IL-1β suggests that the protein is correctly folded. See Hazuda, *et al.* (1969) J. Biol. Chem. 264:1689-1693.

V. Biological Assays with mouse IGIF

Biological assays confirmed the IFN-γ inducing activity on T cells. IGIF stimulates production of IFN-γ by purified NK cells, and that induction is strongly synergized with IL-12 or IL-2. Similar assays will be performed with human IL-1γ.

Mouse IGIF does not appear to function efficiently on human cell, e.g., through the human receptor.

Mouse IGIF has been established to induce IFN-γ by splenocytes from SCID mice, as described above. The splenocytes thus express a receptor for mouse IGIF. The induction also is synergized with IL-12 or IL-2.

Mouse IGIF, in combination with IL-12 (and TNF-α) also induced IL-2 activated NK cells to produce IFN-γ. This induction, in the case of IL-2 activated NK cells, appears to be IL-1β dependent, as treatment with anti-IL-1β blocks the IFN-γ production. The mouse IGIF can overcome this block and induce IFN-γ

VI. Preparation of antibodies specific for IL-1γ

Inbred Balb/c mice are immunized intraperitoneally with recombinant forms of the human protein, e.g., purified soluble IL-1γ FLAG or stable transfected NIH-3T3 cells. Animals are boosted at appropriate time points with protein, with or without additional adjuvant, to further stimulate antibody production. Serum is collected, or hybridomas produced with harvested spleens.

Alternatively, Balb/c mice are immunized with cells transformed with the gene or fragments thereof, either endogenous or exogenous cells, or with isolated membranes enriched for expression of the antigen. Serum is collected at the appropriate time, typically after numerous further administrations. Various gene therapy techniques may be useful, e.g., in producing protein in situ, for generating an immune response.

Monoclonal antibodies may be made. For example, splenocytes are fused with an appropriate fusion partner and hybridomas are selected in growth medium by standard procedures. Hybridoma supernatants are screened for the presence of antibodies which bind to the human IL-1γ, e.g., by ELISA or other assay. Antibodies which specifically recognize human IL-1γ but not species variants may also be selected or prepared.

In another method, synthetic peptides or purified protein are presented to an immune system to generate monoclonal or polyclonal antibodies. See, e.g., Coligan (1991) Current Protocols in Immunology Wiley/Greene; and Harlow and Lane (1989) Antibodies: A Laboratory Manual Cold Spring Harbor Press. In appropriate situations, the binding reagent is either labeled as described above, e.g., fluorescence or otherwise, or immobilized to a substrate for panning methods. Nucleic acids may also be introduced into cells in an animal to produce the antigen, which serves to elicit an immune response. See, e.g., Wang, *et al.* (1993) Proc. Nat'l. Acad. Sci. 90:4156-4160; Barry, *et al.* (1994) BioTechniques 16:616-619; and Xiang, *et al.* (1995) Immunity 2: 129-135.

VII. Production of fusion proteins with IL-1γ

Various fusion constructs are made with IL-1γ. This portion of the gene is fused to an epitope tag, e.g., a FLAG tag, or to a two hybrid system construct. See, e.g., Fields and Song (1989) Nature 340:245-246.

The epitope tag may be used in an expression cloning procedure with detection with anti-FLAG antibodies to detect a binding partner, e.g., receptor for the IL-1γ. The two hybrid system may also be used to isolate proteins which specifically bind to IL-1γ.

VIII. Mapping of human IL-1γ by in situ hybridization

Chromosome spreads are prepared. In situ hybridization is performed on chromosome preparations obtained from phytohemagglutinin-stimulated human lymphocytes cultured for 72 h. 5-bromodeoxyuridine is added for the final seven hours of culture (60 µg/ml of medium), to ensure a posthybridization chromosomal banding of good quality.

An appropriate fragment, e.g., a PCR fragment, amplified with the help of primers on total B cell cDNA template, is cloned into an appropriate vector. The vector is labeled by nick-translation with ^{3}H. The radiolabeled probe is hybridized to metaphase spreads as described in Mattei, *et al.* (1985) Hum. Genet. 69:327-331.

After coating with nuclear track emulsion (KODAK NTB_2), slides are exposed, e.g., for 18 days at 4° C. To avoid any slipping of silver grains during the banding procedure, chromosome spreads are first stained with buffered Giemsa solution and metaphase photographed. R-banding is then performed by the fluorochrome-photolysis-Giemsa (FPG) method and metaphases rephotographed before analysis.

IX. Structure activity relationship

Information on the criticality of particular residues is determined using standard procedures and analysis. Standard mutagenesis analysis is performed, e.g., by generating many different variants at determined positions, e.g., at the positions identified above, and evaluating biological activities of the variants. This may be performed to the extent of determining positions which modify activity, or to focus on specific positions to determine the residues which can be substituted to either retain, block, or modulate biological activity.

Alternatively, analysis of natural variants can indicate what positions tolerate natural mutations. This may result from populational analysis of variation among individuals, or across strains or species. Samples from selected individuals are analysed, e.g., by PCR analysis and sequencing. This allows evaluation of population polymorphisms.

X. Isolation of a Receptor for Human IL-1γ

A human IL-1γ can be used as a specific binding reagent to identify its binding partner, by taking advantage of its specificity of binding, much like an antibody would be used. A binding reagent is either labeled as described above, e.g., fluorescence or otherwise, or immobilized to a substrate for panning methods.

The binding composition is used to screen an expression library made from a cell line which expresses a binding partner, i.e. receptor. Standard staining techniques are used to detect or sort intracellular or surface expressed receptor, or surface expressing transformed cells are screened by panning. Screening of intracellular expression is performed by various staining or immunofluorescence procedures. See also McMahan, *et al.* (1991) EMBO J. 10:2821-2832.

For example, on day 0, precoat 2-chamber permanox slides with 1 ml per chamber of fibronectin, 10 ng/ml in PBS, for 30 min at room temperature. Rinse once with PBS. Then plate COS cells at 2-3 x 10^5 cells per chamber in 1.5 ml of growth media. Incubate overnight at 37° C.

On day 1 for each sample, prepare 0.5 ml of a solution of 66 µg/ml DEAE-dextran, 66 µM chloroquine, and 4 µg DNA in serum free DME. For each set, a positive control is prepared, e.g., of human IL-1γ-FLAG cDNA at 1 and 1/200 dilution, and a negative mock. Rinse cells with serum free DME. Add the DNA solution and incubate 5 hr at 37° C. Remove the medium and add 0.5 ml 10% DMSO in DME for 2.5 min. Remove and wash once with DME. Add 1.5 ml growth medium and incubate overnight.

On day 2, change the medium. On days 3 or 4, the cells are fixed and stained. Rinse the cells twice with Hank's Buffered Saline Solution (HBSS) and fix in 4% paraformaldehyde (PFA)/glucose for 5 min. Wash 3X with HBSS. The slides may be stored at -80° C after all liquid is removed. For each chamber, 0.5 ml incubations are performed as follows. Add HBSS/saponin (0.1%) with 32 µl/ml of 1 M NaN_3 for 20 min. Cells are then washed with HBSS/saponin 1X. Add human IL-1γ or IL-1γ/antibody complex to cells and incubate for 30 min. Wash cells twice with HBSS/saponin. If appropriate, add first antibody for 30 min. Add second antibody, e.g., Vector anti-mouse antibody, at 1/200 dilution, and incubate for 30 min. Prepare ELISA solution, e.g., Vector Elite ABC horseradish peroxidase solution, and preincubate for 30 min. Use, e.g., 1 drop of solution A (avidin) and 1 drop solution B (biotin) per 2.5 ml HBSS/saponin. Wash cells twice with HBSS/saponin. Add ABC HRP solution and incubate for 30 min. Wash cells twice with HBSS, second wash for 2 min, which closes cells. Then add Vector diaminobenzoic acid (DAB) for 5 to 10 min. Use 2 drops of buffer plus 4 drops DAB plus 2 drops of H_2O_2 per 5 ml of glass distilled water. Carefully remove chamber and rinse slide in water. Air dry for a few minutes, then

add 1 drop of Crystal Mount and a cover slip. Bake for 5 min at 85-90° C.

Evaluate positive staining of pools and progressively subclone to isolation of single genes responsible for the binding.

Alternatively, IL-1γ reagents are used to affinity purify or sort out cells expressing a receptor. See, e.g., Sambrook, *et al.* or Ausubel, *et al.*

Another strategy is to screen for a membrane bound receptor by panning. The receptor cDNA is constructed as described above. The ligand can be immobilized and used to immobilize expressing cells. Immobilization may be achieved by use of appropriate antibodies which recognize, e.g., a FLAG sequence of a IL-1γ fusion construct, or by use of antibodies raised against the first antibodies. Recursive cycles of selection and amplification lead to enrichment of appropriate clones and eventual isolation of receptor expressing clones.

Phage expression libraries can be screened by human IL-1γ. Appropriate label techniques, e.g., anti-FLAG antibodies, will allow specific labeling of appropriate clones.

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled; and the invention is not to be limited by the specific embodiments that have been presented herein by way of example.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Schering Corporation
(B) STREET: 2000 Galloping Hill Road
(C) CITY: Kenilworth
(D) STATE: New Jersey
(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): 07033-0530
(G) TELEPHONE: 908-298-2906
(H) TELEFAX: 908-298-5388
(I) TELEX:

(ii) TITLE OF INVENTION: Human Cytokine

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Macintosh
(C) OPERATING SYSTEM: Macintosh 7.5.3
(D) SOFTWARE: Microsoft Word 5.1a

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/651,998
(B) FILING DATE: 20-MAY-1996

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1380 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 435..1016

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AAAGAGATAC TCAGAAAGAG GTACAGGTTT TGGAAGGCAC AGAGCCCCAA CTTTTACGGA      60

AGAAAAGATT TCATGAAAAT AGTGATATTA CATTAAAAGA AGTACTCGTA TCCTCTGCCA     120

CTTTATTTCG ACTTCCATTG CCCTAGGAAA GAGCCTGTTT GAAGGCGGGC CCAAGGAGTG     180

CCGACAGCAG TCTCCTCCCT CCACCTTCTT CCTCATTCTC TCCCCAGCTT GCTGAGCCCT     240

TTGCTCCCCT GGCGACTGCC TGGACAGTCA GCAAGGAATT GTCTCCCAGT GCATTTTGCC     300

CTCCTGGCTG CCAACTCTGG CTGCTAAAGC GGCTGCCACC TGCTGCAGTC TACACAGCTT     360

CGGGAAGAAG AAAGGAACCT CAGACCTTCC AGATCGCTTC CTCTCGCAAC AAACTATTTG     420

TCGCCAGAAT AAAG ATG GCT GCT GAA CCA GTA GAA GAC AAT TGC ATC AAC        470
                Met Ala Ala Glu Pro Val Glu Asp Asn Cys Ile Asn
                  1               5                  10

TTT GTG GCA ATG AAA TTT ATT GAC AAT ACG CTT TAC TTT ATA GCT GAA       518
Phe Val Ala Met Lys Phe Ile Asp Asn Thr Leu Tyr Phe Ile Ala Glu
        15                  20                  25

GAT GAT GAA AAC CTG GAA TCA GAT TAC TTT GGC AAG CTT GAA TCT AAA       566
Asp Asp Glu Asn Leu Glu Ser Asp Tyr Phe Gly Lys Leu Glu Ser Lys
    30                  35                  40

TTA TCA GTC ATA AGA AAT TTG AAT GAC CAA GTT CTC TTC ATT GAC CAA       614
Leu Ser Val Ile Arg Asn Leu Asn Asp Gln Val Leu Phe Ile Asp Gln
45                  50                  55                  60

GGA AAT CGG CCT CTA TTT GAA GAT ATG ACT GAT TCT GAC TGT AGA GAT       662
Gly Asn Arg Pro Leu Phe Glu Asp Met Thr Asp Ser Asp Cys Arg Asp
                65                  70                  75

AAT GCA CCC CGG ACC ATA TTT ATT ATA AGT ATG TAT AAA GAT AGC CAG       710
Asn Ala Pro Arg Thr Ile Phe Ile Ile Ser Met Tyr Lys Asp Ser Gln
            80                  85                  90

CCT AGA GGT ATG GCT GTA ACT ATC TCT GTG AAG TGT GAG AAA ATT TCA       758
Pro Arg Gly Met Ala Val Thr Ile Ser Val Lys Cys Glu Lys Ile Ser
        95                  100                 105

ACT CTC TCC TGT GAG AAC AAA ATT ATT TCC TTT AAG GAA ATG AAT CCT       806
Thr Leu Ser Cys Glu Asn Lys Ile Ile Ser Phe Lys Glu Met Asn Pro
    110                 115                 120

CCT GAT AAC ATC AAG GAT ACA AAA AGT GAC ATC ATA TTC TTT CAG AGA       854
Pro Asp Asn Ile Lys Asp Thr Lys Ser Asp Ile Ile Phe Phe Gln Arg
125                 130                 135                 140
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AGT GTC CCA GGA CAT GAT AAT AAG ATG CAA TTT GAA TCT TCA TCA TAC      902
Ser Val Pro Gly His Asp Asn Lys Met Gln Phe Glu Ser Ser Ser Tyr
                145                 150                 155

GAA GGA TAC TTT CTA GCT TGT GAA AAA GAG AGA GAC CTT TTT AAA CTC      950
Glu Gly Tyr Phe Leu Ala Cys Glu Lys Glu Arg Asp Leu Phe Lys Leu
            160                 165                 170

ATT TTG AAA AAA GAG GAT GAA TTG GGG GAT AGA TCT ATA ATG TTC ACT      998
Ile Leu Lys Lys Glu Asp Glu Leu Gly Asp Arg Ser Ile Met Phe Thr
        175                 180                 185

GTT CAA AAC GAA GAC TAG CTATTAAAAT TTCATGCCGG GCGCAGTGGC            1046
Val Gln Asn Glu Asp
    190

TCACGCCTGT AATCCCAGCC CTTTGGGAGG CTGAGGCGGG CAGATCACCA GAGGTCAGGT   1106

GTTCAAGACC AGCCTGACCA ACATGGTGAA ACCTCATCTC TACTAAAAAT ACAAAAAATT   1166

AGCTGAGTGT AGTGACCCAT GCCCTCAATC CCAGCTACTC AAGAGGCTGA GGCAGGAGAA   1226

TCACTTGCAC TCCGGAGGTG GAGGTTGTGG TGAGCCGAGA TTGCACCATT GCGCTCTAGC   1286

CTGGGCAACA ACAGCAAAAC TCCATCTCAA AAAATAAAAT AAATAAATAA ACAAATAAAA   1346

AATTCATAAT GTGAAAAAAA AAAAAAAAAA AAAG                               1380
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 194 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Ala Ala Glu Pro Val Glu Asp Asn Cys Ile Asn Phe Val Ala Met
 1               5                  10                  15

Lys Phe Ile Asp Asn Thr Leu Tyr Phe Ile Ala Glu Asp Asp Glu Asn
            20                  25                  30

Leu Glu Ser Asp Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile
        35                  40                  45

Arg Asn Leu Asn Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro
    50                  55                  60

Leu Phe Glu Asp Met Thr Asp Ser Asp Cys Arg Asp Asn Ala Pro Arg
65                  70                  75                  80

Thr Ile Phe Ile Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met
                85                  90                  95

Ala Val Thr Ile Ser Val Lys Cys Glu Lys Ile Ser Thr Leu Ser Cys
            100                 105                 110
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Glu Asn Lys Ile Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile
        115                 120                 125

Lys Asp Thr Lys Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly
    130                 135                 140

His Asp Asn Lys Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe
145                 150                 155                 160

Leu Ala Cys Glu Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys
                165                 170                 175

Glu Asp Glu Leu Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu
            180                 185                 190

Asp
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WHAT IS CLAIMED IS:

1. An antagonist of human IL-1γ.

2. The antagonist of claim 1 which comprises an antibody against human IL-1γ, or a binding fragment thereof.

3. A pharmaceutical composition for inhibiting the biological activity of human IL-1γ comprising an antagonist of either claim 1 or 2 and a pharmaceutically acceptable carrier.

4. A method for treating a condition caused by human IL-1-γ comprising administering an effective amount of an antagonist or pharmaceutical composition of any one of claims 1 to 3 to an individual in need of such treatment.

5. The use of an antagonist of either claim 1 or 2 for the preparation of a medicament for inhibiting the biological activity of human IL-1γ.

6. The use of an antagonist of either claim 1 or 2 for treating a condition caused by human IL-1γ.

7. An isolated human IL-1γ receptor.

8. A fusion protein comprising human IL-1γ covalently conjugated to polyethylene glycol or to a polypeptide.

9. The fusion protein of claim 8 in which a polypeptide conjugated to the human IL-1γ is derived from an immunoglobulin chain, preferably an Fc fragment, or is another cytokine or chemokine.

10. A pharmaceutical composition for supplying the biological activity of human IL-1γ comprising a fusion protein of either claim 8 or 9 and a pharmaceutically acceptable carrier.

11. A method for supplying the biological activity of human IL-1γ comprising administering an effective amount of a fusion protein or pharmaceutical composition of any one of claims 8 to 10 to an individual in need of such activity.

12. The use of a fusion protein of either claim 8 or 9 for the preparation of a medicament for supplying the biological activity of human IL-1γ.

13. The use of a fusion protein of either claim 8 or 9 for supplying the biological activity of human IL-1γ.

14. An isolated nucleic acid or vector encoding a fusion protein of either claim 8 or 9.

15. A method for producing a fusion protein comprising human IL-1γ covalently conjugated to a polypeptide, comprising culturing a host cell comprising the nucleic acid or vector of claim 14 under conditions in which the nucleic acid or vector is expressed.

16. An anti-idiotypic antibody which is an agonist or antagonist of human IL-1γ.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/07282

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C07K16/24 A61K39/395 C07K14/715 C07K19/00
A61K38/20 C07K16/42

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NATURE, vol. 379, no. 6566, 15 February 1996, LONDON, GB, page 591 XP002039798 J. BAZAN ET AL.: "A newly defined interleukin-1?" cited in the application see the whole document ---	1-16
A	PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH. ANNUAL MEETINGS, vol. 37, no. 0, March 1996, USA, page 452 XP002039799 K. TORIGOE ET AL.: "Structure and function of a novel human IFN-gamma inducing factor (HuIGIF)." see abstract #3083 --- -/--	1-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 September 1997	25.09.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Nooij, F

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INTERNATIONAL SEARCH REPORT

Intern. ial Application No
PCT/US 97/07282

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 267 611 A (OTSUKA PHARMACEUTICAL CO.) 18 May 1988 see examples see claims	1-16
A	NATURE, vol. 378, no. 6552, 2 November 1995, LONDON, GB, pages 88-91, XP002039800 H. OKAMURA ET AL.: "Cloning of a new cytokine that induces IFN-gamma production by T cells." cited in the application see page 88, right-hand column, line 34 - line 36 see figures 1A,2B	1-16
P,A	THE JOURNAL OF IMMUNOLOGY, vol. 156, no. 11, 1 June 1996, BALTIMORE, MD, USA, pages 4274-4279, XP002039801 S. USHIO ET AL.: "Cloning of the cDNA for human IFN-gamma-inducing factor, expression in Escherichia coli, and studies on the biologic activities of the protein." see page 4279, left-hand column, line 4 - line 9 see figure 1	1-16
P,A	EP 0 712 931 A (KABUSHIKI KAISHA HAYASHIBARA SEIBUTSU KAGAKU KENKYUJO) 22 May 1996 see examples see sequences see claims	1-16

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INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US 97/07282

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 4,6,11, and 13
because they relate to subject matter not required to be searched by this Authority, namely:

remark: Although claim(s) 4,6,11 (complete); 13 (partially) is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 97/07282

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 267611 A	18-05-88	DE 3785994 A	01-07-93
		DE 3785994 T	11-11-93
		ES 2054644 T	16-08-94
		JP 6339394 A	13-12-94
		JP 2003205 C	20-12-95
		JP 7020438 B	08-03-95
		JP 63258595 A	26-10-88
EP 712931 A	22-05-96	JP 8193098 A	30-07-96
		JP 8231598 A	10-09-96
		AU 3779695 A	23-05-96
		CA 2162353 A	16-05-96